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CONFORMATIONAL ORDER IN LIPID TUBULES FORMED FROM A  
DIACETYLENIC LECITHIN: A RAMAN SPECTROSCOPIC STUDY(U)  
NAVAL RESEARCH LAB WASHINGTON DC J P SHERIDAN

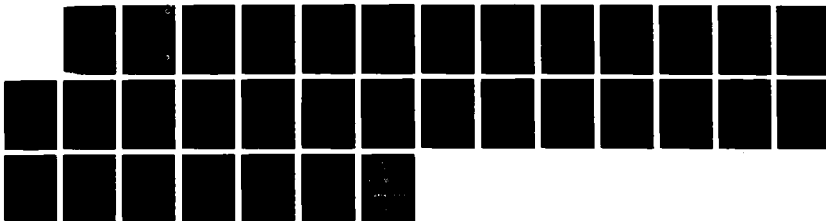
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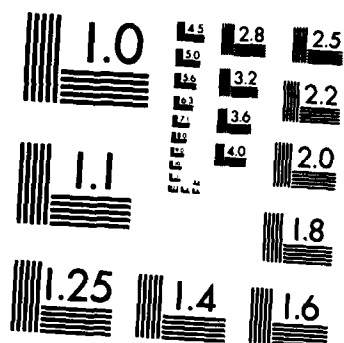
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**Conformational Order in Lipid Tubules  
Formed From a Diacetylenic Lecithin:  
A Raman Spectroscopic Study**

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*Bio/Molecular Engineering Branch  
Chemistry Division*

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| <p>The conformational characteristics of the monomeric lipids in the novel tubular structure formed from the diacetylenic phosphatidylcholine DC8,9PC have been determined by laser Raman spectroscopy. It is found that DC8,9PC tubules are generally much more crystalline than other lipid microstructures such as liposomes characterized at the same reduced temperature and it is suggested that this high degree of lipid chain ordering may be a key factor in the driving force for tubule formation. In particular, it is found that the diacetylenic unit decouples the two halves of the acyl chains into approximately <i>m</i> methylene segments (upper half of the lipid) and (<i>n</i>+diacetylenic unit) segments (lower half of the lipid molecules). The <i>m</i> portion of the chains is always fully extended in the all-<i>trans</i> conformations in freshly prepared tubes as determined by observation of a strong longitudinal acoustic mode, while the <i>n</i> segments are initially somewhat less well</p> |   |           |  |                |                                 |                            |
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ordered. However, a very high degree of order can be induced by annealing the tubules at low temperature, resulting in both halves of the chains adopting a rigid, fully extended all-*trans* conformation which is then maintained upon returning to room temperature. This is further confirmed quantitatively by analysis of one of the skeletal optical modes in terms of the fraction of *trans* bonds present. In addition, it is determined that laterally adjacent lipids in thermally annealed tubules are packed very closely next to each other, as are the terminal methyl portions of lipids in apposing monolayers, and that the crystal lattice sub-cell is probably orthorhombic or monoclinic.

## CONTENTS

|                               |    |
|-------------------------------|----|
| INTRODUCTION .....            | 1  |
| EXPERIMENTAL PROCEDURES ..... | 4  |
| RESULTS AND DISCUSSION .....  | 6  |
| CONCLUSIONS .....             | 17 |
| ACKNOWLEDGEMENTS .....        | 18 |
| REFERENCES .....              | 26 |

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# CONFORMATIONAL ORDER IN LIPID TUBULES FORMED FROM A DIACETYLENIC LECITHIN: A RAMAN SPECTROSCOPIC STUDY

## INTRODUCTION

It has been known for over two decades (Bangham et al., 1965) that phosphatidylcholine lipids self-organize into spherical structures consisting of an aqueous space surrounded by single or multiple lipid bilayers with dimensions on the micron and submicron scale. These phospholipid "vesicles" or "liposomes" have been extensively investigated because of their usefulness as models for biological membranes and their potential application as carriers for drug delivery. In an effort to enhance vesicle stability for the latter application, as well as many other biotechnological possibilities, phospholipids containing polymerizable moieties such as diacetylene, vinyl, methylacryloyl and butadiene functional groups have been synthesized (O'Brien et al., 1985 and references within). Liposomes composed of such lipids can be polymerized by irradiation or by chemical initiation, often resulting in enhanced stability towards physical and chemical perturbations (Regan et al., 1982; Hub et al., 1980; Johnston et al., 1980; O'Brien et al., 1981; Tundo et al., 1982).

In most cases the monomeric lipids containing such polymerizable groups were found to form spheroidal bilayer microstructures which were topologically stable both above and below the lipid chain melting temperature ( $T_m$ ). However, recently it was discovered (Yager and Schoen, 1984) that aqueous dispersions of a phosphatidylcholine containing diacetylene groups formed hollow cylindrical structures in the low temperature phase.

The polymerizable phospholipid, 1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine (DC8,9PC), is a phosphatidylcholine that contains a diacetylene group halfway down each of its 23-carbon hydrocarbon chains (see Figure 1). DC8,9PC has a single broad phase transition centered around 42C on heating and 38C on cooling. Yager and Schoen (1984) observed that, above T<sub>m</sub>, the lipid forms stable liposomes, but as the temperature is dropped the liposomes change their morphology in a manner dependant on the rate of cooling. If the fluid-phase large multilamellar vesicles (MLVs) composed of this lipid were cooled rapidly to below 30C, they became unstable and broke violently into small "shards", but if the temperature is lowered slowly (<1C/min) and the liposomes are larger than 1µm, they converted quantitatively to a low temperature phase with unusual cylindrical morphology. The microstructures, which they referred to as tubules, consisted of a large cylindrical aqueous core surrounded by a number of bilayers (Yager et al., 1985). The tubules had outer diameters that ranged from 0.3 to 1 µm and lengths of up to hundreds of micrometers depending upon conditions of formation (Yager and Schoen, 1984).

Because the cylindrical shape of tubules offers a new variable to exploit in biotechnological applications of lipid microstructures, the experimental conditions and molecular features of DC8,9PC responsible for tubule formation are of fundamental interest. Clearly, if we know why tubes form, we will then have a good handle on the types of useful lipid modifications which can be effected (while still retaining tubule integrity) such as introduction of liquid crystal groups, chemically active head groups, improvements in stability, control of dimensions, etc. - all of which would enhance our ability to tailor



tubules for specific biotechnological applications. However, the problem of tubule formation is an extremely difficult one and it is likely that it can only be solved by a close coupling of a wide range of experimental methods, theory, and molecular modeling.

A number of the issues concerning tubule structure and stability revolve around questions of chain conformation and packing and the extent to which this might be important in tubule formation. Such questions can be addressed experimentally by spectroscopic tools. Raman spectroscopy is one such physical tool which has proven valuable in probing the structural properties of lipid bilayer phases (Levin, 1984; Yager and Gaber, 1986 and references contained within). Information concerning lateral chain-chain interactions, conformational disorder, hydration effects on the head group, as well as indications of crystal sub-cell geometry and the presence of chain interdigitation are examples of the type of data that can be extracted from the Raman spectra of lipids. In particular, a quantitative method of elucidating the fraction of *trans* or *gauche* bonds along the acyl chains and a semiquantitative method of estimating the degree of lateral packing order between chains in saturated PC's from Raman line intensities have recently been developed (Sheridan, 1986; Gaber and Peticolas, 1977).

In their initial characterization of DC8,9PC, Schoen and Yager (1985) conducted infrared and Raman studies on the various phases exhibited by the diacetylenic lipid. Their infrared data suggested that the DC8,9PC lipids in the low temperature tubule morphology were packed in a manner similar to that found in the sub-gel phase of dipalmitoyl PC (Cameron and Mantsch, 1982).

However, they were unable to confirm this quantitatively by Raman because the experiments were conducted with 514.5nm laser light which caused fairly rapid polymerization of the diacetylene groups in the low temperature phase, resulting in a marked increase in fluorescence and resonance enhanced bands which masked to a large degree many of the most conformationally sensitive spontaneous Raman bands. However, the use of a longer wavelength excitation source (such as the 647.1nm line of a krypton laser) can aid in overcoming the unwanted polymerization initiation and allow a more quantitative analysis of chain conformation and packing of the DC8,9PC monomeric lipids in tubular morphology. Thus, in order to further our understanding of the differences at the molecular level between tubular and liposomal bilayer structure, we present in this report the results of a krypton laser Raman spectroscopic study of the low temperature phases exhibited by DC8,9PC and compare them where appropriate, with those obtained for its saturated PC analogue, 1,2-ditricosanoyl-sn-glycero-3-phosphocholine (DTPC) in aqueous suspension.

## EXPERIMENTAL PROCEDURES

**Materials.** The lipid, 1,2-bis(10,12-tricosadiynoyl)-sn-glycero-3-phosphocholine was synthesized in our laboratory using previously published methodologies (Johnston et al., 1980; Singh and Schnur, 1986) and purified to a single spot by thin layer chromatography on silica gel (E. Merck) using a chloroform/methanol/water (65:25:4) solvent system. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-ditricosanoyl-sn-glycero-3-phosphocholine were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL) and were used without further purification.

**Preparation of Aqueous Lipid Suspensions.** Lipid suspensions were prepared in the following manner: Lipid films were prepared in glass tubes by drying the lipid from chloroform under nitrogen. The remaining solvent was

removed by placing the sample under high vacuum for 12 hours. Distilled water was added to the film to yield a lipid concentration of 100 mg/ml. Hydration of the lipid was achieved by maintaining the sample above  $T_m$  for two hours without agitation. Tubule suspensions were prepared by cooling the hydrated lipid sample at a rate of 1 degree per minute through the chain melting transition. Presence of tubules was confirmed in freshly prepared tubule samples by examination at room temperature via light microscopy. Those samples containing a high yield of tubules were then pelleted at room temperature in glass capillaries using a hematocrit centrifuge spinning at 13,000 rpm. The capillaries were then heat sealed and stored in the dark at room temperature or at 4C until used.

**Raman Spectroscopy.** The sample capillaries were held in a copper cell which was thermostatted by a temperature-controlled ethylene glycol-water solution circulating from a Neslab RTE-8 bath. The temperature was monitored near the sample site by a thermocouple and temperature control was generally found to be on the order of 0.1°C or better. The copper sample holder was mounted within a plexiglass chamber through which flowed a constant stream of dry Argon in order to prevent condensation at low temperatures. The chamber was equipped with glass windows to allow entry of the laser beam and 90° collection of the scattered light. The excitation source was the 647.1nm line of a Krypton ion laser. Laser power incident upon the sample was usually between 150mW and 200mW. This power range was particularly critical for DC23PC samples as power levels much above 200mW usually resulted in partial photopolymerization of the sample after a few hours exposure to the laser beam (presumably due to two photon absorption). Raman spectra were recorded using a Spex 14018 double monochromator equipped with holographic

gratings, a cooled RCA 31034 photomultiplier, and photon counting detection system. The spectrometer slits were generally set at 600  $\mu\text{m}$  except for the low frequency experiments, where slit settings of 200  $\mu\text{m}$  were used. The spectrometer was controlled by a Spex Datamate DM1B minicomputer system.

## RESULTS AND DISCUSSION

### Low Frequency Region

The low frequency region of a Raman spectrum (practically speaking  $\sim 50$ - $500\text{ cm}^{-1}$ ) contains bands due to collective motions of the crystal lattice (if one exists) and to overall vibrations of the whole molecules. The simplest, but most conformationally sensitive of these for a hydrocarbon chain molecule is the longitudinal acoustic mode (LAM) which is an accordion-like vibration of fully extended chain segments. Thus, it is a highly specific probe of the existence of chain segments in the all-trans conformation and its frequency is inversely proportionate to the length of the all-trans sequences (Schaufele and Shimanouchi, 1967; Peticolas, 1979). Observation of such a mode, its frequency, and its relative integrated intensity, can yield important information about the degree of conformational order in any given phase of the chain molecule under investigation.

Figure 2 shows the Raman spectra in the low frequency region for polycrystalline samples of DC8,9PC and its homologue, DC8,13PC. Two relatively intense bands are observed in both spectra. One band at  $262\text{ cm}^{-1}$  is common to both homologues while the other occurs at  $196\text{ cm}^{-1}$  for DC8,9PC and at  $146\text{ cm}^{-1}$  for DC8,13PC. We tentatively attribute these bands to the LAMs of all-trans segments of the upper and lower halves of the paraffinic chains on either side of the diacetylene group in the two lipids. The length

of these sequences can in principle be estimated from the formula:

$$\nu_{\text{LAM}} = 2 \nu_0 \sin (\pi/N) \quad (1)$$

Here  $\nu_0$  is  $375 \text{ cm}^{-1}$  and  $N$  is the number of carbon atoms in the all-trans configuration. As  $\pi/N$  is much less than 1 we can develop (1) in a Taylor series to give:

$$\nu_{\text{LAM}} = 2356 \text{ cm}^{-1}/N \quad (2)$$

This formula was derived by Peticolas (1979) for fatty acids and fits well with experimental data for such molecules. The extent to which (2) can be used to estimate all-trans sequences in diacetylenic phospholipids is currently unknown and its accuracy may well be affected by end effects and the presence of the diacetylene moiety. Nevertheless, it is instructive to use (2) to make at least a rough estimate of the all-trans segments in DC8,9PC and DC8,13PC. Application of (2) to the observed low frequency modes in these lipids yield the following values of  $N$ :

|          | $\nu \text{ (cm}^{-1}\text{)}$ | $N$ |
|----------|--------------------------------|-----|
| DC8,9PC  | 262                            | 9   |
|          | 196                            | 12  |
| DC8,13PC | 262                            | 9   |
|          | 146                            | 16  |

A number of interesting observations may be made concerning these data:

1) The estimated value of  $m$  (in the generic  $DCm,nPC$  nomenclature) is identical for both lipids and close to that expected if the  $m$  segments are indeed all-trans (9 vs 8). 2) The estimated value of  $n$  for both lipids appears to be approximately equal to  $n+3$  where  $n$  is the actual number of methylenes in the lower chain segment of each lipid. A possible explanation for this may be that the  $n$  chain segment is vibrationally coupling to the diacetylene moiety. Another possible reason may be that end effects are more important for the  $n$  segment than for the  $m$  segment, i.e. the  $n$  segment sees a "wall" consisting of the rest of the lipid molecule. The net result would be a decrease in the LAM frequency from that expected for a free chain of the same length. 3) The difference between the estimated  $n$  values for the two lipids is 4 - exactly the difference between the actual lengths of the  $n$  segments (13 vs 9) for 8,9PC and 8,13PC. This last point is of major importance in that the frequency shift anticipated in the LAM for a change in  $n$  of four carbons is indeed experimentally observed. This lends strong support for the assignment of the observed low frequency modes to LAMs of the upper and lower halves of the fatty acyl chains on either side of the diacetylene moiety.

The upper trace in Figure 3 shows the low frequency Raman spectrum of a sample of tubules prepared from DC8,9PC and held at 20C directly after having been slowly cooled through  $T_m$ . As in the case of the polycrystalline solid, two low frequency modes can be observed at about  $196\text{ cm}^{-1}$  and  $262\text{ cm}^{-1}$ . However, in the case of the freshly prepared tubules, the mode at  $196\text{ cm}^{-1}$  is somewhat broader and weaker than that observed in the solid lipid.

while the  $262\text{ cm}^{-1}$  mode is about as intense as that in the solid. This suggests that, in the freshly prepared tubules, the upper half of the acyl chains are all in the fully extended all-trans conformation while some fraction of the lower chain segments are less well conformationally ordered. This interpretation will be confirmed from analysis of skeletal vibrations discussed in the next section.

The lower trace in Figure 3 shows a Raman spectrum of the same tubule preparation after having been annealed at 0C for about two days. It can be clearly seen that the LAM at  $196\text{ cm}^{-1}$  has now become sharper and increased in intensity relative to the mode at  $262\text{ cm}^{-1}$  indicating that, after annealing at low temperature, the acyl chains in the lower half of the lipid molecules have "crystallized" into their fully extended all-trans conformation. This is qualitatively similar to the annealing process observed in the formation of the Lc phase in saturated-chain phosphatidylcholines. However, the LAM modes observed in DC8,9PC tubules are much more intense than any ever before seen in lipids -- even in polycrystalline PCs. Thus the low temperature tubule phase of DC8,9PC appears to possess the highest degree of acyl chain conformational order ever encountered in a lamellar lipid bilayer. Furthermore, once this highly ordered structure is formed at low temperature it remains locked in for at least 20 degrees on being allowed to return to room temperature. It has been demonstrated that the symmetric C-N stretch in phosphatidylcholines can be used as an internal standard for relative intensity measurements (Gaber and Peticolas, 1977). Thus, the relative intensities of the two LAM modes in DC8,9PC tubules, recorded at several temperatures, were normalized to the symmetric C-N stretch at  $718\text{ cm}^{-1}$  and are listed below:

| Temperature      | I196/I718 | I262/I718 |
|------------------|-----------|-----------|
| 20C (unannealed) | 0.85      | 3.0       |
| 0C (annealed)    | 1.1       | 3.0       |
| 10C (annealed)   | 1.1       | 3.0       |
| 20C (annealed)   | 1.08      | 3.0       |
| 30C (annealed)   | 0.7       | 2.2       |
| 40C (annealed)   | 0         | 0.4       |

Inspection of the data shown above reveals that both halves of the acyl chains remain fully extended in the all-trans conformation through 20C after annealing at 0C. From 20C through 40C, the lower acyl chain segment becomes progressively more disordered at a rate faster than that found for the upper segment. Spectra taken at 45C, above the chain melting temperature, show no sign of either of the two LAMs (data not shown), consistent with the expectation that the fluid-state bilayer phase would have a fairly high percentage of gauche bonds resulting in a very low probability for the existence of all-trans chain segments as long as 8 or 9 carbons.

#### Skeletal Region

Figure 4A depicts the Raman spectrum in the range  $650\text{ cm}^{-1}$  -  $1550\text{ cm}^{-1}$  for DC8,9PC tubules recorded at 0C. The sample of tubules had been equilibrated at this temperature for several hours. It is typical of a bilayer phase possessing highly ordered chains. The skeletal optical modes (SOM) at  $1064\text{ cm}^{-1}$  and  $1128\text{ cm}^{-1}$  are sharp and intense reflecting the presence of rigid all-trans polymethylene chains. These SOM bands have been identified as the in-plane and out-of-plane C-C stretching modes of trans segments arising from the sum of pairs of trans bonds involving at least three contiguous



carbons (Lippert and Peticolas, 1971). Two additional relatively sharp bands are observed at  $1070\text{ cm}^{-1}$  and  $1092\text{ cm}^{-1}$  respectively. Vibrational transitions at almost identical frequencies have been observed for saturated fatty acids with  $n=10$  and  $n=14$  (Lippert and Peticolas, 1972). Such transitions have been assigned to the  $k=1$  C-C stretching mode for an all-trans hydrocarbon chain (Lippert and Peticolas, 1972). Furthermore, they have been shown to be a sensitive function of chain length for a series of fatty acids in the range  $n=10$  and  $n=24$ , decreasing in frequency as the chain length shortens (Lippert and Peticolas, 1972). The two sharp transitions at  $1070\text{ cm}^{-1}$  and  $1092\text{ cm}^{-1}$  in the spectrum of DC23PC correlate with those observed for  $n=10$  and  $n=14$  respectively in the fatty acid series studied by Lippert and Peticolas. This is suggestive of the existence of two all-trans acyl chains containing 8 and 12 trans bonds respectively in the DC8,9PC tubules. This is also fairly consistent with the LAM data discussed in the previous section which provided evidence that the hydrocarbon chains in DC8,9PC were vibrationally decoupled into two portions.

The band near  $890\text{ cm}^{-1}$  is the methyl deformation mode for the conformer  $\text{CH}_3\text{-CH}_2^{\text{T}}\text{-CH}_2^{\text{T}}\text{-CH}_2$ , ie. with two trans bonds adjacent to the methyl group (Zerbi et al., 1981a,b), while the one at  $875\text{ cm}^{-1}$  is the C-N antisymmetric stretch. If gauche conformers are present near the ends of the acyl chains, two other bands appear at about  $866\text{ cm}^{-1}$  and  $844\text{ cm}^{-1}$  corresponding respectively to the  $\text{CH}_3\text{-CH}_2^{\text{G}}\text{-CH}_2^{\text{T}}\text{-CH}_2$  and  $\text{CH}_3\text{-CH}_2^{\text{T}}\text{-CH}_2^{\text{G}}\text{-CH}_2$  conformers (Zerbi et al., 1981a,b). Thus, these bands are specific markers of order/disorder near the terminal methyl groups of the acyl chains. Inspection of this region of the spectrum in Fig. 1A reveals that the band near  $890\text{ cm}^{-1}$  is well resolved and more intense than the C-N antisymmetric stretch at  $875\text{ cm}^{-1}$  while there is little evidence of bands in the  $845 - 870\text{ cm}^{-1}$  region. The C-N symmetric stretch around  $720\text{ cm}^{-1}$  has

been shown previously (Gaber and Peticolas, 1977) to be useful as an internal intensity standard in phospholipid bilayers. Thus, the integrated intensity of the methyl deformation mode was measured and normalized against the  $720\text{ cm}^{-1}$  band. A value of 0.38 was obtained which can be compared with the value of 0.35 obtained for the sub-gel ( $L_c$ ) phase of DPPC (Sheridan, unpublished data). As was the case with the LAM data discussed in the previous section, the relative intensity of the methyl deformation mode remained essentially constant through  $20^\circ\text{C}$  and then began to drop slowly in intensity as the temperature was further raised, indicating that defects were being introduced in the region of the chain termini. This, again, is consistent with the thermally-induced changes observed in the intensity of the LAM associated with the lower acyl chain segments. Furthermore, the frequency of the methyl deformation mode was found to be  $893\text{ cm}^{-1}$ . This may be compared with a value of  $892\text{ cm}^{-1}$  found in the  $L_c$  phase of DPPC. A similar value is found for the orthorhombic crystal phase of n-alkanes (Zerbi et al., 1981b), a phase in which the opposing lamellae, and therefore the methyl groups, are very tightly packed against each other. Inspection of the methylene deformation region ( $1400\text{--}1500\text{ cm}^{-1}$ ) in Figure 4A reveals a band at  $1420\text{ cm}^{-1}$  which is, in fact, characteristic of alkyl chains in an orthorhombic subcell (Boerio and Koenig, 1970). This orthorhombic signature remains strong right up to the chain melting temperature. Thus, it would appear that, in the low-temperature-annealed tubules, the tendency of acyl chains in an orthorhombic lattice to pack together as tightly as possible is manifested at the bilayer mid-plane with the methyl groups of one lipid monolayer packing tightly against the methyl groups in the apposing monolayer, and that this persists over a fairly wide temperature range (compared with other lipid bilayer systems).

By way of contrast, shown in Figure 4B is the skeletal region spectrum of DC8,9PC's saturated analogue, DTPC, recorded at the same reduced temperature as that for DC8,9PC. Close inspection of Figure 4b reveals a number of significant differences from the skeletal spectrum of DC8,9PC. The SOM at  $1128\text{ cm}^{-1}$  is weaker in intensity than that for DC8,9PC and there exists only one relatively weak  $k=1$  C-C mode at a frequency commensurate with that for a chain containing about 20 trans bonds. Again, the methyl deformation mode is found at a frequency of  $890\text{ cm}^{-1}$  rather than  $892\text{ cm}^{-1}$ , and it is considerably weaker than its counterpart in the spectrum of DC8,9PC. Its relative intensity (normalized against the symmetric CN stretch) is found to be 0.25 compared with 0.38 for DC8,9PC. Finally, there is no evidence of an orthorhombic packing signature in the  $1420\text{ cm}^{-1}$  region. Thus the picture which emerges from this spectrum of DTPC is one in which the lattice is less well packed and there are some gauche defects in the chains, particularly in the region of the chain termini at the bilayer mid-plane.

#### Quantitative Measure of Conformational Order

The most useful quantitative measure of conformational order in the interior of a lipid bilayer is the fraction of bonds in the hydrocarbon chains which are trans or gauche. Although the LAMs are the most conformationally sensitive bands, they are typically weak in lipid dispersions and are difficult to measure being easily obscured by strong background and water scattering. The SOM bands around  $1064\text{ cm}^{-1}$  and  $1130\text{ cm}^{-1}$ , also representative of all-trans hydrocarbon segments, have been more frequently used as relative measures of trans-bond content in lipid bilayers. Various means of internal normalization have been used (Gaber and Peticolas, 1977; Susi et al., 1980; Vogel and Jahnig, 1981). However, none of them have proven satisfactory when attempts were made to use the normalized ratios as quantitative measures of conformational

order in lipids. Recently, Strobl (1978) has convincingly demonstrated that the total integrated intensity of the methylene (CH<sub>2</sub>) twisting mode, centered around 1295cm<sup>-1</sup> in the solid and 1300cm<sup>-1</sup> in the melt, is relatively insensitive to phase or temperature in the case of polyethylene. Using this band as his internal standard, he was able to use the Raman technique to determine the degree of crystallinity in partially crystalline polyethylene and obtained results that were in excellent agreement with those derived from other physical methods (Strobl and Hagedorn, 1978). Thus, in this work, the total integrated intensity of the CH<sub>2</sub> twisting mode has been chosen as the internal reference standard. Using this mode, a quantitative scale of molecular order (or fluidity) can be established, based on the integrated intensity ratios of I<sub>1130</sub>/I<sub>1295</sub> in the crystalline and fluid phases of linear n-alkanes, which can then be used to estimate the fraction of trans bonds in any lipid bilayer phase containing saturated acyl chains. The detailed description of the methodology is the subject of another report (Sheridan, submitted for publication, 1986). Briefly, in the crystalline phases of a linear n-alkane such as n-nonadecane the chains are in the all-trans or nearly all-trans conformation, while in the fluid phase the chains are in an isotropic environment, so that the trans bond populations at various temperatures can be readily calculated using the methods described by Flory (1969). This then allows a calibration of the Raman intensity ratios as functions of conformational structure. Such a calibration curve is shown in Figure 5 where the intensity ratio I<sub>1130</sub>/I<sub>1295</sub> is plotted as a function of trans bond population.

The total integrated intensities of the 1130 cm<sup>-1</sup> modes of both DC8,9PC tubules and DC8,9PC shards were measured for samples which had been incubated at their respective reduced temperatures of (T<sub>m</sub>-40)C for two days. These intensities were normalized over that of the CH<sub>2</sub> twisting mode and the respective fractions of trans bonds estimated from the calibration curve in

Figure 5. The Ftrans values so obtained are shown in Table 1. The same procedure was adopted for the saturated chain analogue, DTPC, and the Ftrans value measured is listed in Table 1. Also listed for comparison are the values for the orthorhombic and hexagonal phases of a simple linear alkane. Inspection of the Ftrans data in Table 1 reveals that the acyl chains in DC8,9PC tubules, which have been annealed at (Tm-40)C, possess the highest fraction of trans bonds - 100% - equal to that exhibited by the orthorhombic phase of an n-alkane. On the other hand, its saturated analogue, DTPC, has a significant fraction of gauche bonds at the same reduced temperature-equivalent to about two gauche bonds per chain. The data for DC8,9PC shards also indicate the presence of some degree of conformational disorder in contrast to the tubule sample annealed under identical conditions. Either the shard fragments have many broken edges containing disordered lipid which do not "heal" under the annealing conditions, or the lipids in the small shard fragments show no propensity towards "crystallizing" into more highly ordered entities.

### C-H Stretching Region

Figure 6A shows the C-H manifold ( $2750\text{cm}^{-1}$  -  $3050\text{cm}^{-1}$ ) recorded at 0C for DC8,9PC after annealing for an extended period of time. There are three bands of particular interest in this region of the spectrum. The symmetric and anti-symmetric C-H stretching modes of the polymethylene chains near  $2850\text{cm}^{-1}$  and  $2880\text{cm}^{-1}$  respectively, and the band centered around  $2935\text{cm}^{-1}$  which is a composite of two overlapping bands, one from the polymethylene chains ( $2922\text{cm}^{-1}$ ) and one from the terminal methyl groups ( $2938\text{cm}^{-1}$ ). The peak-height intensity ratios  $I_{2880}/I_{2850}$  and  $I_{2935}/I_{2880}$  have been widely used as indices of lateral hydrocarbon chain-chain interactions (Gaber and

Peticolas, 1977; Huang et al., 1982; Wong and Mantsch, 1983). However, in addition to those peaks discussed above, the C-H stretching manifold for DC8,9PC possesses additional peaks not seen in any saturated lipid or hydrocarbon. In particular, there is a sharp peak at  $2910\text{ cm}^{-1}$  and a shoulder on the high frequency side of the  $2886\text{ cm}^{-1}$  peak to complicate matters. As has been suggested by Schoen and Yager, it may be that the Fermi resonance-factor group splitting of the hydrocarbon chains is quite different in diacetylenic lipids, or that the diacetylenic group electronically perturbs neighboring methylenes. Clearly these additional spectral features in the C-H stretching region of DC8,9PC complicate efforts to analyze the intensity ratios in terms of those derived from saturated lipids. Nevertheless, it is useful for comparative purposes to convert some of the C-H data into the form employed by Gaber and Peticolas (1977). They defined a measure of lateral order (Slat) in terms of the ratio of peak heights  $I_{2886}/I_{2850}$  ( $r$ ) as follows:

$$\text{Slat} = (r - 0.7)/1.5 \quad (3)$$

so that  $S = 1$  for a chain in a close packed orthorhombic crystal ( $r = 2.2$  for the orthorhombic phase of *n*-alkanes such as nonadecane), and  $S = 0$  for a chain in the fluid state ( $r = 0.7$  for the liquid phase of *n*-alkanes).

Applying the formalism in (3) to the case of DC8,9PC, Slat values were calculated for annealed tubules and shards at  $(T_m-40)^\circ\text{C}$  and are listed in Table I together with the analogous value for the fully saturated chain lipid, DTPC, also measured at  $(T_m-40)^\circ\text{C}$ . As before, values for the orthorhombic and hexagonal phases of *n*-nonadecane are included for comparison.

Inspection of the Slat data in Table I again indicates that the DC8,9PC tubules consist of lipids which are very highly ordered. In this case the data

reflect how tightly packed the chains are in the tubule lattice. Comparison with the Slat value for DTPC at the same reduced temperature shows that they are very tightly packed indeed. The value for DC8,9PC lies close to that for the orthorhombic phase of n-nonadecane, while that for DTPC is considerably less than the value recorded for the more loosely packed hexagonal phase of n-C19. In fact the Slat value for the annealed DC8,9PC tubules is higher than any ever recorded for a 1,2-diacyl-phosphatidylcholine lipid.

### CONCLUSIONS

Based on the preceding analysis of the Raman data for DC8,9PC tubules and saturated chain DTPC liposomes the following conclusions can reasonably be stated:

- 1) DC8,9PC tubules appear to be generally much more crystalline than other lipid microstructures composed of saturated chain phosphatidylcholines. The crystal lattice present is probably orthorhombic or monoclinic.
- 2) The acyl chains are decoupled into two segments of approximately  $m$  carbons and  $(n+\text{diacetylene unit})$  carbons. The  $n$  segments of the chains are initially less well ordered than the  $m$  segments in thermally grown tubes, but a high degree of order can be induced by annealing at low temperature, resulting in both segments adopting an extended all-trans conformation in the annealed tubules.
- 3) The existence of the all-trans acyl chain conformation in the low-temperature-annealed tubules is confirmed quantitatively by analysis of the normalized intensity of the SOM at  $1130\text{ cm}^{-1}$ .
- 4) Laterally adjacent lipids in the annealed tubules are packed very closely next each other.
- 5) The terminal methyl groups of one monolayer in the tubule bilayer appear to be packed tightly against those of the lipids in the apposing monolayer.

6) The vibrational signatures of tubules and shards appear to be qualitatively similar to one another. However, there appears to exist some degree of conformational and lateral disorder in the shards, even after annealing at low temperature.

7) The high degree of lipid chain ordering is a probably a key factor in the driving force for tubule formation. Thus, any modification of the diacetylenic lipids for novel applications should be made in such a way that their inherent propensity towards high crystallinity, and thus tubule formation, is maintained.

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TABLE 1

| LIPID                | TEMPERATURE              | F <sub>trans</sub> | S <sub>lat</sub> |
|----------------------|--------------------------|--------------------|------------------|
| DC8,9PC<br>(TUBULES) | (T <sub>M</sub> - 40)°C  | 1.0                | 0.83             |
| DC8,9PC<br>(SHARDS)  | (T <sub>M</sub> - 40)°C) | 0.93               | 0.71             |
| DTPC                 | (T <sub>M</sub> - 40)°C. | 0.85               | 0.41             |
| n-C19                | 20°C (ORTHO.)            | 1.0                | 1.0              |
| "                    | 35°C (HEXAG.)            | 0.96               | 0.60             |

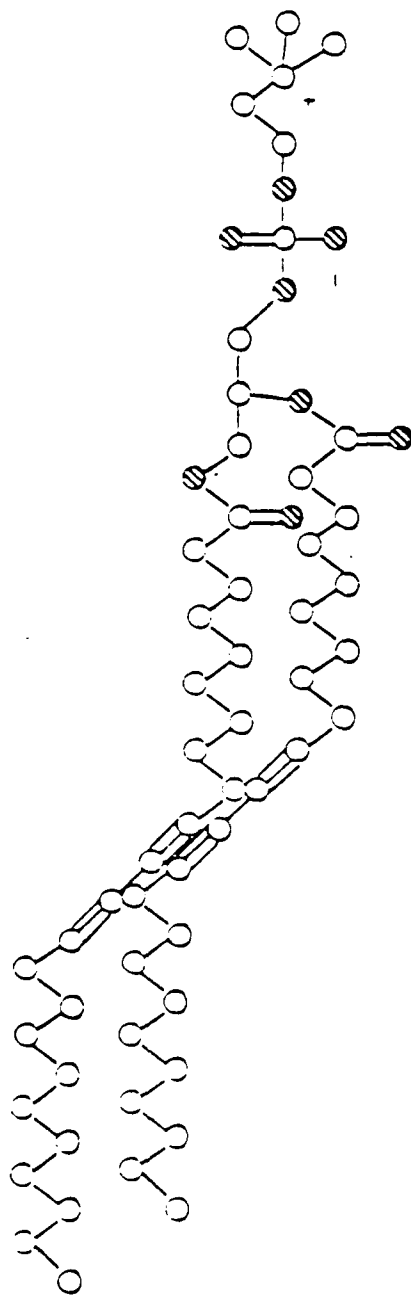
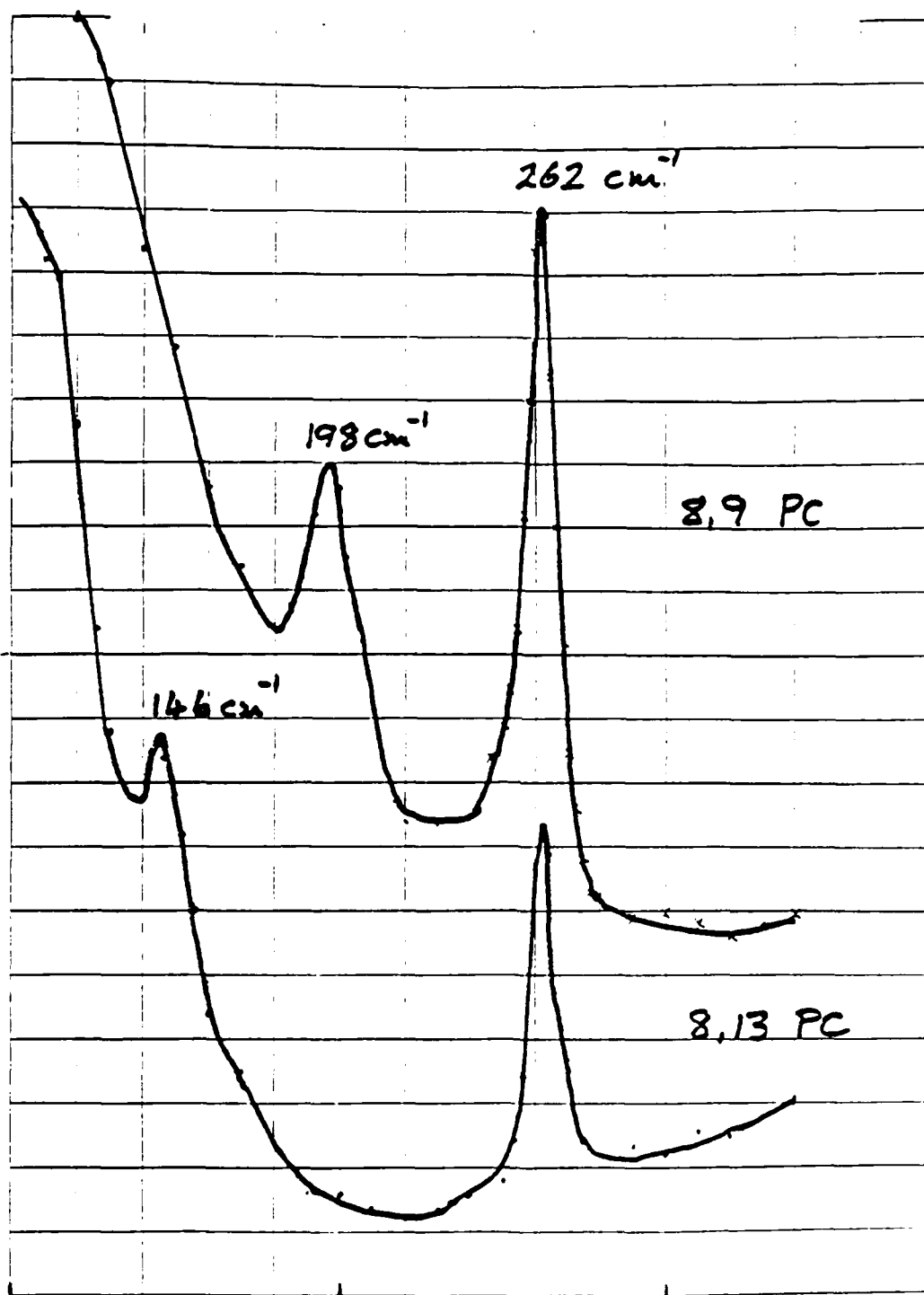


Figure 1. Schematic drawing of DC8,9PC.



**Figure 2.** Raman spectra recorded in the low frequency region for polycrystalline samples of DC8,9PC (upper trace), and DC8,13PC (lower trace).

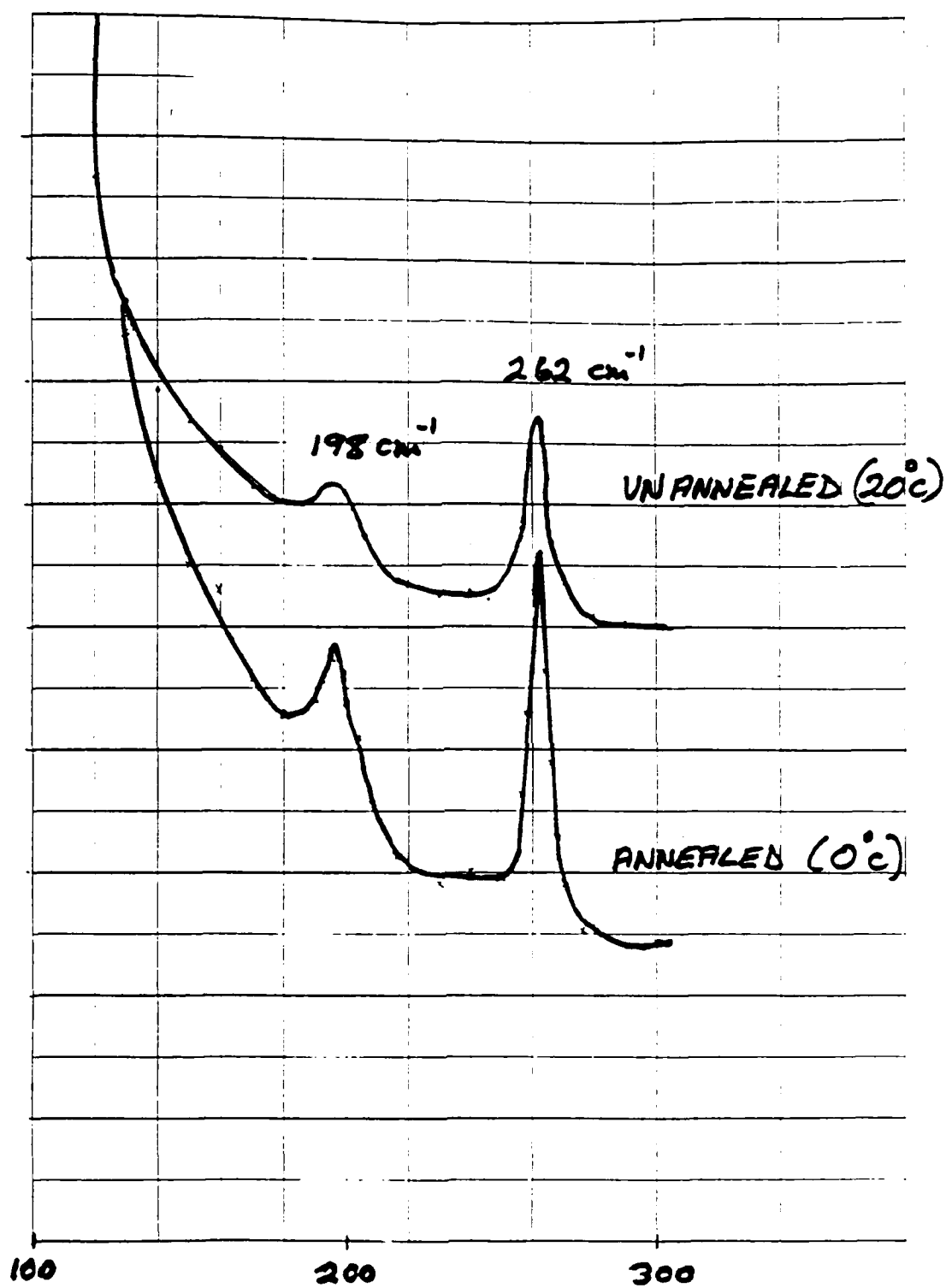
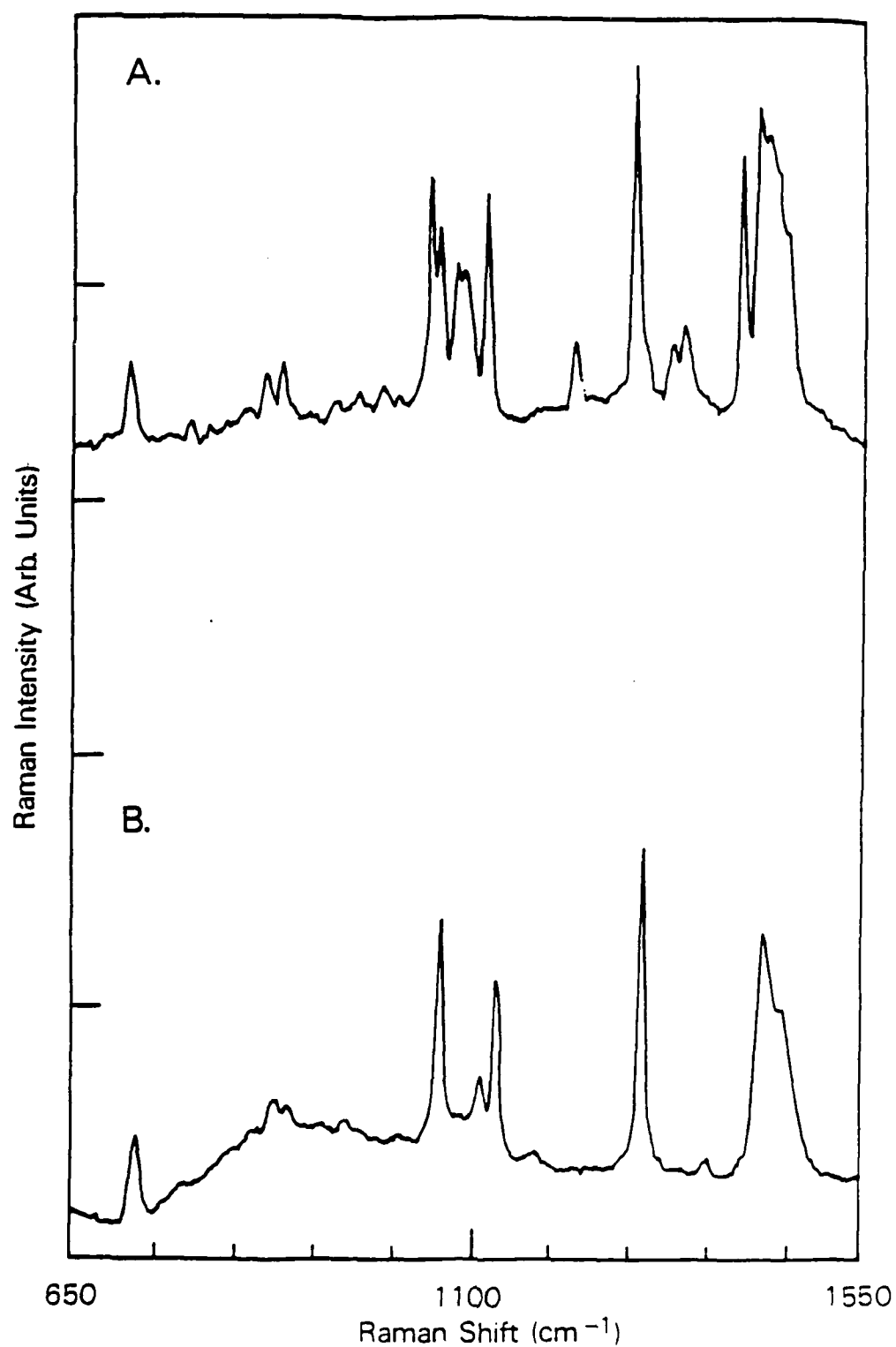
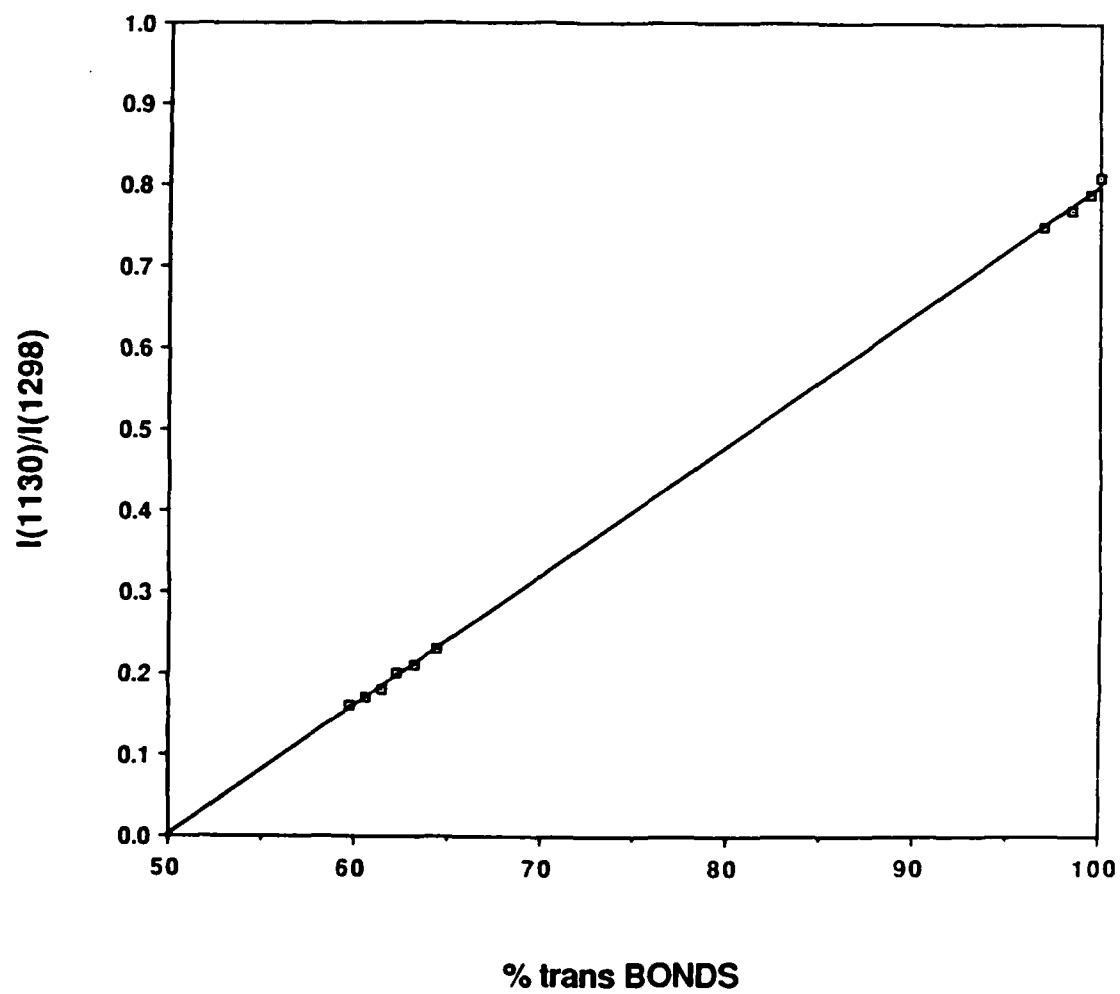


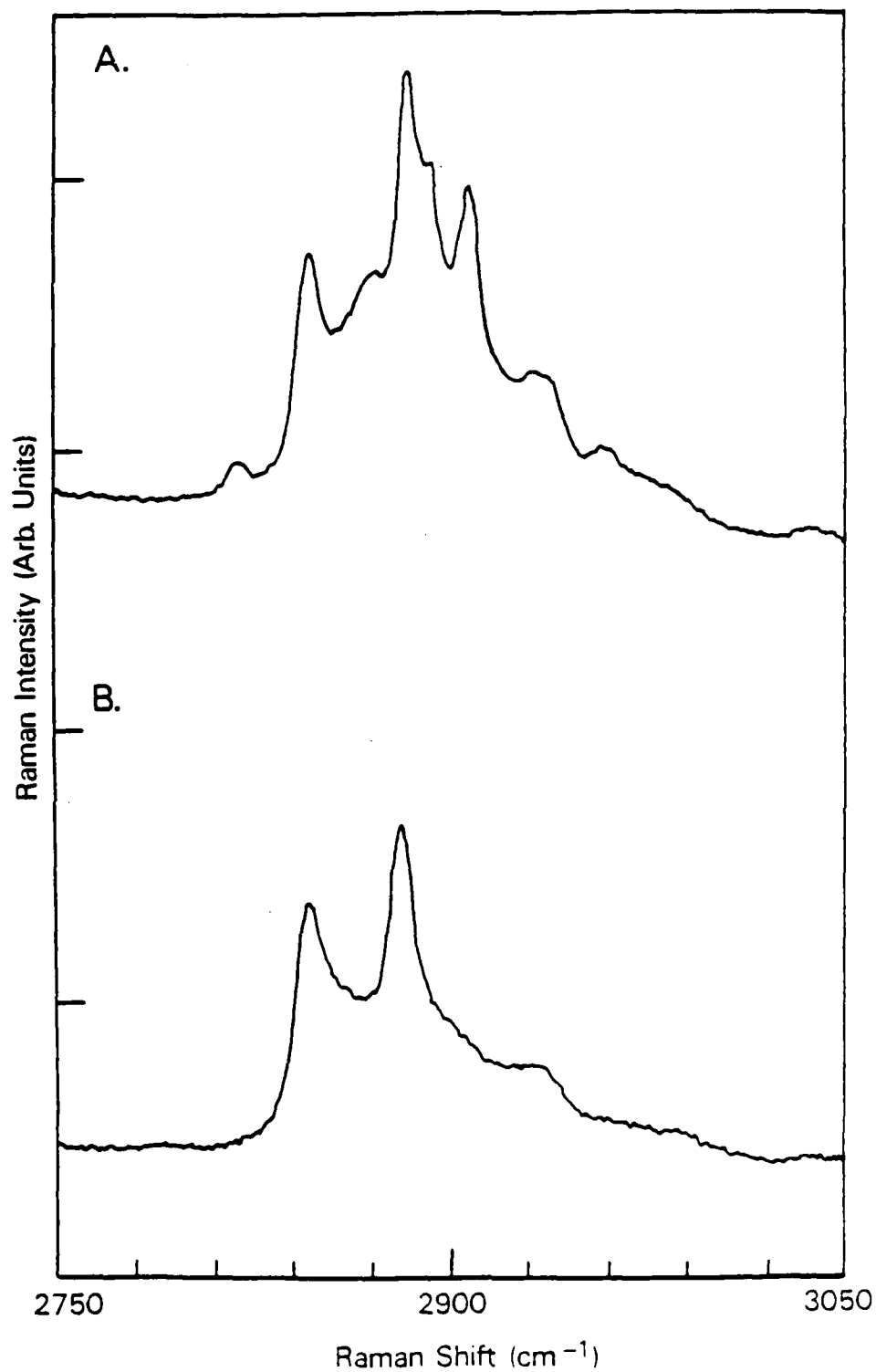
Figure 3. Raman spectra recorded in the low frequency region for DC3.9PC tubules at 20°C (upper trace), and at 0°C after two days annealing (lower trace).



**Figure 4.** Raman spectra recorded in the skeletal region between 650cm<sup>-1</sup> and 1550 cm<sup>-1</sup>: A. DC8,9PC tubules after annealing at 0C; B. DTPC liposomes at the same reduced temperature, (T<sub>m</sub>-40)C.



**Figure 5.** Plot of Integrated intensity ratio  $I(1130)/I(1295)$  against percentage of trans bonds.



**Figure 6.** Raman spectra recorded in the C-H stretching region between 2750cm<sup>-1</sup> and 3050 cm<sup>-1</sup>: A. DC8,9PC tubules after annealing at 0C: B. DTPC liposomes at the same reduced temperature, (T<sub>m</sub>-40)C.

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